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Pleiotropic Effects of HTLV Type 1 Tax Protein on Cellular Metabolism: Mitotic Checkpoint Abrogation and NF-κB Activation

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ABSTRACT

Tax protein expressed by human T cell leukemia virus type 1 (HTLV-1) is a strong *trans*-activator of its own LTR promoter; it also affects the function of multiple cellular genes involved in cell cycle control and transcription. One way in which Tax exerts its pleiotropic effects is through protein–protein interaction with cellular cofactors. By using yeast two-hybrid technology, we have isolated several cellular proteins that bind to Tax. Two of these are MAD1, a mitotic checkpoint control protein, and TXBP151, a suppressor of tumor necrosis factor α-induced apoptosis. Here we discuss findings describing the role of MAD1 in exit of cells from mitosis and TXBP151 in NF-κB activation.

INTRODUCTION

Human T cell leukemia virus type 1 (HTLV-1) is the etiological agent for adult T-cell leukemia (ATL)[1,2] and neurological disorders termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[3] ATL develops in a minority of HTLV-1-infected individuals, and has a 20- to 30-year latent period. This disease course suggests a virus-induced multistage process of immortalization and transformation of T lymphocytes. Ultimately, ATL results from the malignant clonal expansion of an HTLV-1-infected T cell, after years of progressive accumulation of genetic lesions, including chromosomal abnormalities and tumor suppressor gene inactivation.[4–10]

HTLV-1 encodes a 40-kDa phosphoprotein, Tax. Tax immortalizes T lymphocytes[11] and transforms rat fibroblasts.[12] Tax is also an activator of HTLV-1 long terminal repeat (LTR)-directed transcription.[13] While the exact events leading to transformation are incompletely understood, several important cellular processes are deregulated by Tax: Tax activates genes responsive to NF-κB,[14] serum response factor (SRF),[15] and CREB/ATF (cAMP-responsive element-binding protein/activating transcription factor)[16,17] and accelerates cell cycle progression.[18,19] Previously it was understood that Tax could influence, via p53, the G_1-to-S phase transition, and the DNA damage sentinel at this juncture[20–23]; however, it was unclear how to account for aneuploidy, polyploidy, and the induction of kinetochore-containing micronuclei in Tax-expressing cells.[24]

Because Tax does not bind directly to DNA, it likely interacts with cellular cofactors to exert its pleiotropic effects. In searching for cellular proteins that directly bind to and might be functionally influenced by Tax, we employed the yeast two-hybrid assay[25] and identified several proteins including HsMAD1,[26] TXBP151,[27] G protein pathway suppressor 2 (GPS2),[28] and I-κB kinase γ (IKKγ).[29] HsMAD1 is a human mitotic checkpoint protein involved in a *m*itotic *a*rrest-*d*eficient phenotype (MAD). Expression of either Tax or a *trans*-dominant-negative HsMAD1 results in multinucleated cells, a phenotype consistent with a loss of HsMAD1 function. This observation is also consistent with reports that human colon cancer cells with chromosomal instability are defective in their mitotic checkpoint;[30] and that some breast cancer cells and lung cancer cells have similar defects in their mitotic checkpoint.[31–33] Findings from Tax–HsMAD1 interaction indicate that Tax subverts cellular regulation at the levels of mitosis and possibly cytokinesis.[26] This would explain the frequent observations of multinucleated giant cells and chromosomal abnormalities in

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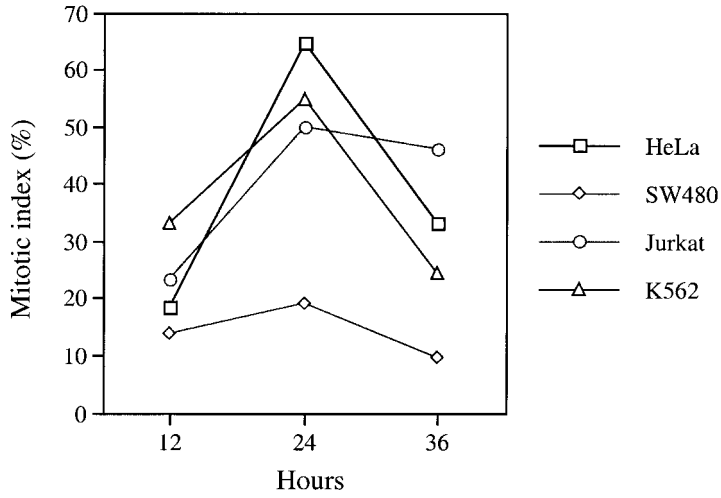

FIG. 1. Mitotic indices of cell lines in the presence of microtubule-depolymerizing agent, nocodazole. A high mitotic index in the presence of nocodazole indicates an intact checkpoint whereas a low mitotic index is consistent with a loss of checkpoint function. Here the profiles of HeLa, Jurkat, and K562 are indicative of the former whereas that of SW480 is compatible with the latter.

cells from ATL patients, and the observation of aneuploidogenic and clastogenic damage in Tax-expressing cells.

To better understand the role of mitotic checkpoint in cellular immortalization, we assessed the mitotic indices[30] of the cell lines HeLa, SW480, Jurkat, and K562. After treatment with nocodazole, significant variations in mitotic indices were found in these cell lines.

Another example of the pleiotropic function of Tax emerged from studies of the zinc finger protein A20.[34] A20 is a primary response gene that was originally identified as a cytokine-inducible gene in human umbilical vein endothelial cells.[35] A20 is also a potent cellular inhibitor of NF-κB activation.[36–39] The Tax-binding protein TXBP151 was unexpectedly found to interact directly with A20.[27] Here we examined the role of TXBP151 in cell survival and apoptosis. We transiently transfected 293T and HeLa cells with either Tax alone or with Tax plus TXBP151 expression vectors and observed a suppressive effect of TXBP151 on Tax-mediated NF-κB activation in both cell lines.

MATERIALS AND METHODS

Cell lines

293T, HeLa, and SW480 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, VT). Jurkat and K562 cells were cultured in RPMI 1640 (BioWhittaker) and supplemented with 10% FBS.

Cell cycle analysis

Nocodazole (Sigma, St. Louis, MO) was added to cells at a final concentration of 0.5 mM. Cells were harvested at 12-hr intervals from 12 to 36 hr. Harvested cell pellets were resuspended in 50 ml of 1% formaldehyde–0.2% glutaraldehyde. Twenty milliliters of the cell suspension was dried on a poly-L-lysine-coated slide and stained with phosphate-buffered saline (PBS) containing (10 μg/ml; Hoechst 33258 Sigma). Fluorescence microscopy was used to assess the mitotic index (percentage of viable cells arrested in mitosis). To measure the mitotic index, at least 300 cells were counted.

NF-κB reporter gene assay

293T and HeLa cells were cultured in 12-well plates to a subconfluent (2×10^5 cells/well) level and transiently transfected using LipofectAMINE (Life Technologies, Bethesda, MD) with 0.5 μg of NF-κB-Cat reporter plasmid (Stratagene, La Jolla, CA), 0.1 μg of CMV-βGAL plasmid, 1 μg of pHpX containing Tax under the control of the HTLV-1 LTR, and with

TABLE 1. MUTATIONS OF HUMAN MITOTIC CHECKPOINT GENES

Gene	*Missense mutation (nucleotide)*	*Tissue*	*Reference*
hsMAD1	G-to-A at codon 299	Lung cancer	33
hsBUB1	G-to-A at codon 140	Colon cancer	30
	C-to-A at codon 492	Colon cancer	30
	197-bp deletion	Colon cancer	30
	A-to-G at codon 950	Colon cancer	42
hsBUBR1	C-to-T at codon 40	Colon cancer	30
	T deletion at codon 1023	Colon cancer	30

or without 1 μg of pCAGGS-TXBP151L.[27] After 36 to 40 hr, cells were washed once with 1× PBS (pH 7.4), resuspended in 200 μl of 250 mM Tris-HCl (pH 8.0), and lysed by three freeze–thaws. Cell lysates were centrifuged for 3 min in a microcentrifuge at 4°C and the supernatant was used for chloramphenicol acetyltransferase (CAT) and β-galactosidase assays.

RESULTS AND DISCUSSION

The phenotypes of the mitotic checkpoint in standard laboratory cell lines are poorly understood. To better comprehend the role of this checkpoint in cellular immortalization we undertook a direct assay of several cell lines. We studied the mitotic indices of HeLa, Jurkat, K562, and SW480 cells when each cell type was exposed to the microtubule-depolymerizing agent nocodazole. HeLa, Jurkat, and K562 cells all had high mitotic indices in the presence of nocodazole. This is consistent with a normal mitotic spindle checkpoint. In contrast, the mitotic index of SW480 cells in the presence of nocodazole was low. This supports the notion that the mitotic checkpoint in SW480 is abnormal, possibly reflecting a more severe degree of transformation (Fig. 1). Consistent with a role of the mitotic checkpoint in cellular immortalization, there have been reports of mutations in human mitotic checkpoint genes in cancer cells (Table 1). Cahill *et al.*[30] examined 19 colorectal cancer cell lines with chromosomal instability and demonstrated that in some, the loss of mitotic checkpoint is associated with the mutational inactivation of the BUB1 (*b*udding *u*ninhibited by *b*enzimidazole) gene. The BUB1 gene is a component of the mitotic checkpoint that delays anaphase in the presence of spindle damage, thus increasing the probability of successful delivery of euploid genome to each daughter cell.[40] Analysis of BUB1 in 31 head-and-neck squamous cell carcinoma and lung cancer cell lines with aneuploidy showed that one nonsense mutation was detected in one cell line.[41] Examination of the MAD1 gene in 49 lung cancer specimens led to the identification of several mutations in MAD1.[32] In the original report of the cloning of human MAD2,[31] a human breast tumor cell line T47D showed sensitivity to taxol and nocodazole and had reduced MAD2 expression. T47D failed to arrest in mitosis after nocodazole treatment. In 32 sporadic digestive cancers examined by Imai *et al.*,[42] mutation of the MAD2 gene was not observed; however, a missense mutation of BUB1 was noted in one rectal cancer.

In our studies, abnormal mitotic indices were observed in several HTLV-1 transformed cell lines (data not shown), suggesting that ATL cells are defective in this checkpoint and are like SW480 cells in being severely transformed. Previously it was shown that a functional target of Tax is the HsMAD1 protein, and that Tax has both aneuploidogenic and clastogenic effects and induces multinuclei in mammalian cells.[26] Therefore, it is possible that the mitotic spindle assembly checkpoint is permanently lost during initiation of transformation in most HTLV-1-transformed cell lines. Karyotypes of 107 cases of ATLL were reviewed by Kamada *et al.* They did not find any

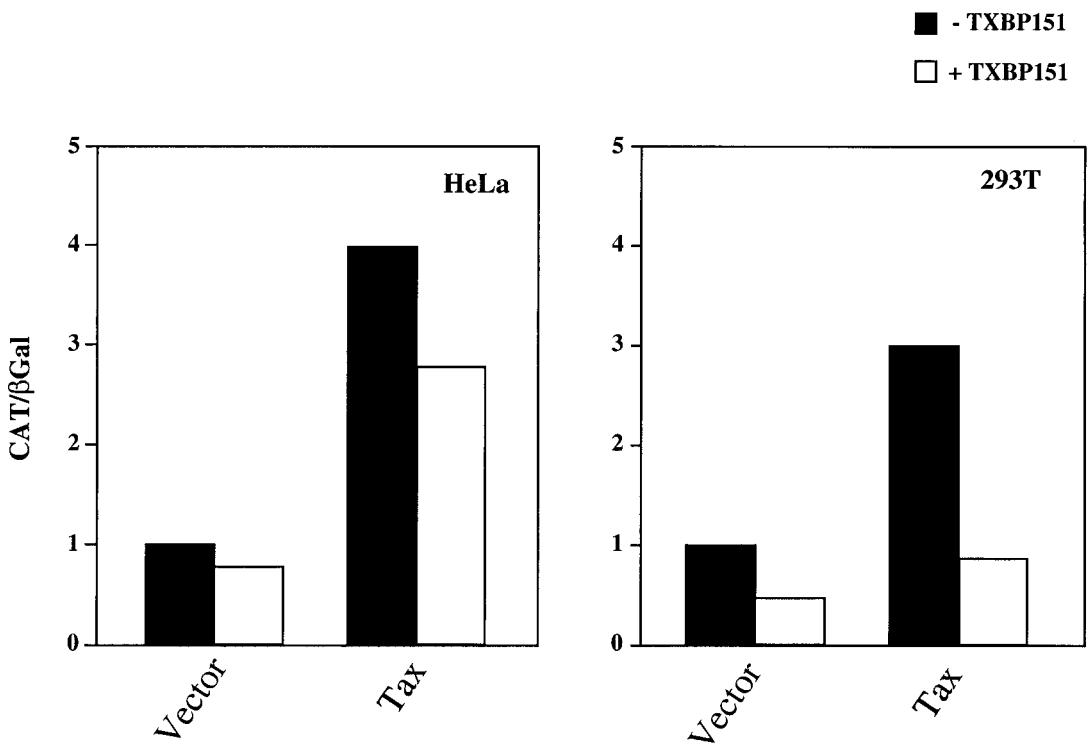

FIG. 2. Suppressive effect of TXBP151 on Tax-mediated activation of NF-κB. HeLa (left) and 293T (right) cells were transiently transfected with a Tax-expressing plasmid, pHpX, either with (open columns) or without (filled columns) 1 μg of TXBP151-expressing plasmid, pCAGGS-Hs151. NF-κB-CAT (0.5 μg) and CMV-βGAL (0.1 μg) receptor plasmids were included in each transfection. Fold activation was based on normalization to β-galactosidase activity. Cells were harvested and assayed for CAT activity after a 40-hr transfection.

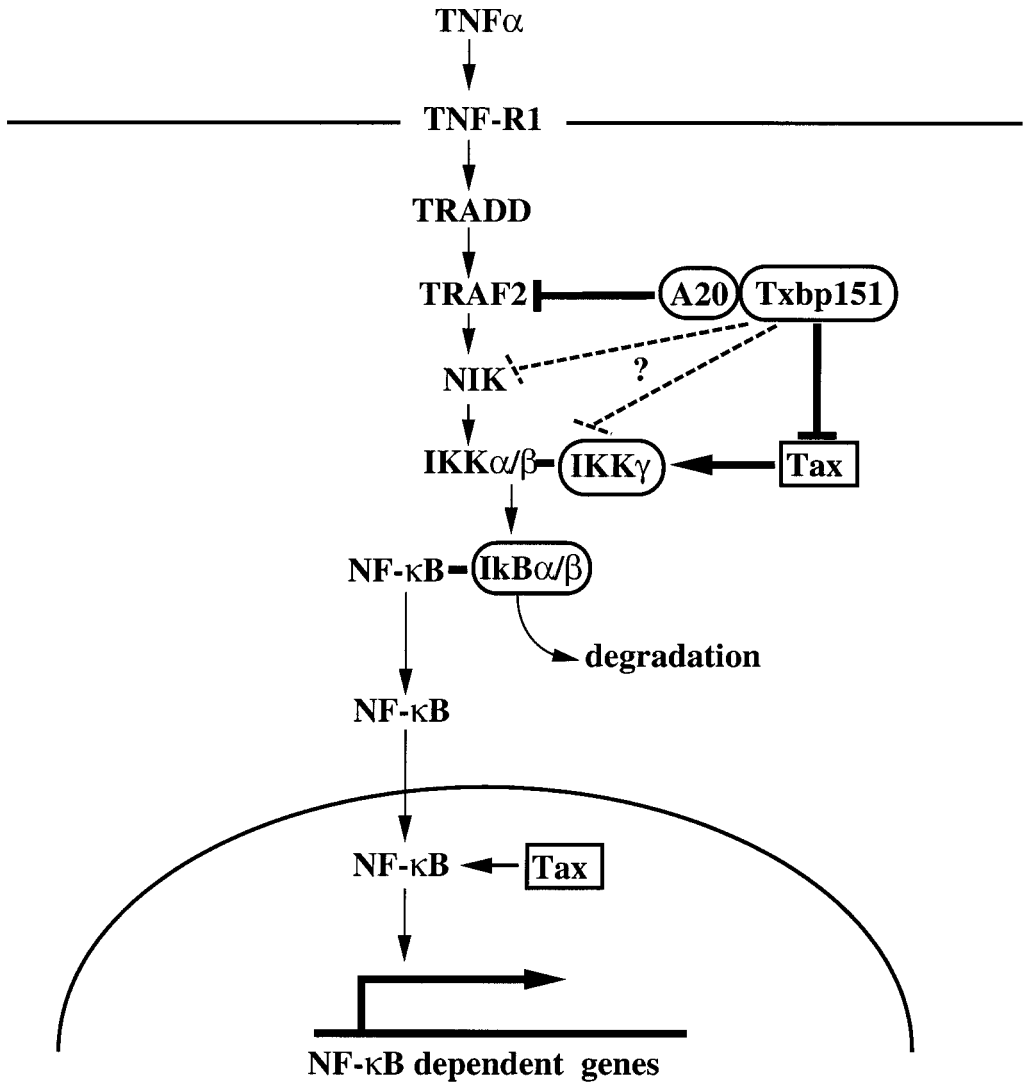

FIG. 3. A Model explaining the role of TXBP151 in the NF-κB activation pathway. Molecules that have a suppressive effect on NF-κB activation are drawn with an oval outline. Abbreviations: TNFα, tumor necrosis factor α; TNFR1, TNF receptor 1; TRADD, TNF receptor-associated death domain protein; TRAF2, TNF receptor-associated factor 2; NIK, nuclear factor κB-inducing kinase; IKK, I-κB kinase; NF-κB, nuclear factor κB.

abnormalities specific to ATL, but showed that most abnormalities appeared more frequently in the aggressive acute or lymphoma type of ATL than in the nonaggressive chronic, or smoldering type of disease.[43]

Transformation of cells by HTLV-1 also involves Tax activation of NF-κB. We also sought to understand how NF-κB might be either positively or negatively regulated in the setting of HTLV-1 Tax. We have previously shown that Tax complexes directly with IKKγ, a component of the IKK complex.[29] This direct interaction with IKKγ correlates with Tax-induced I-κBα phosphorylation and NF-κB activation. In addition to this finding, we have also isolated another Tax-interacting molecule, TXBP151, which is also involved in the signal transduction pathway of NF-κB activation.[27] TXBP151 was originally isolated by two independent groups as an HTLV-1 Tax-binding protein[27,44] and also as an A20-binding protein.[27] Because TXBP151 can bind multiple factors such as A20 and Tax and because it has a suppressive effect on tumor necrosis factor α (TNF-α) induced apoptosis in NIH 3T3 cells, TXBP151 is thought to work at the level between TNF receptor-associated protein 2 (TRAF2) and IKKγ. To assess this hypothesis, we transiently transfected Tax with or without TXBP151 expression plasmids into 293T or HeLa cells (Fig. 2). Tax-mediated *trans*-activation of NF-κB-dependent CAT expression was found to be suppressed by TXBP151 in both cell lines.

We previously described an antiapoptotic activity of TXBP151 on TNF-α treatment of cells. TXBP151 was shown to be cleaved by caspase 3-like proteases during TNF-α- and CD95 (Fas/APO-1)-induced apoptosis. From those results, it could be reasoned that TXBP151 serves a role as a mediator of A20 antiapoptotic activity.[27] Further experiments, to be reported elsewhere, indicate that there are additional roles for TXBP151 beyond that of a mediator of A20 activity. Our cur-

rent model for the involvement of TXBP151 in cellular signaling is summarized in Fig. 3.

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